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SITE-SPECIFIC ANTAGONISTS TO TETRODOTOXIN AND SAXITOXIN

ANNUAL REPORT

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Through studies on structure-activity relations of some natural and synthetic analogs of TTX and STX, and by complementarity considerations, the binding site for these toxins have been deduced as being a pocket 9.5 A (width) x 6 A (height) x 5 A (depth). There are 7 anchoring site points (a - g) in the receptor which interact with the toxin molecules. Past attempts in synthesizing agonists and antagonists of the toxins have focussed on compounds which could bind to sites a (ion-pairing site), b and c (hydrogen-bonding sites). These attempts have not been successful. In the past year, we have attempted to synthesize compounds which could bind to sites a, f and g. Twelve compounds were synthesized and 9 tested. They have ED_{50}'s for blocking sodium channel ranging from 0.5 to 10 mM. All of them also interfer with potassium channels to varying degrees. We continued to collect more 11-oxoTTX for synthesizing a specifically labelled ^{3}HTTX and photoactivatable derivatives of TTX. A photo label, 4-amidopentafluronitrobenzene, has been synthesized, and we are coupling it to 11-oxoTTX. Biological effects of this material remain to be tested.

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INTRODUCTION

Project goals. The objective of this project is to generate more knowledge about the specific chemical structures of the tetrodotoxin (TTX) and saxitoxin (STX) binding site on the voltage-gated sodium-channel protein. It is hoped that from such knowledge, site-specific antagonists to these toxins can be developed rationally. Moreover, identification of the binding site will greatly aid further understanding of the three-dimensional structure of the sodium-channel, and such knowledge will facilitate our understading of the actions of other sodium-channel effectors, and the development of appropriate specific antagonists.

The project has been progressing on two parallel tracks: (a) to expand and refine current knowledge of the structure-activity relations of TTX/STX analogues, and (b) to produce new synthetic compounds which might mimic or block the actions of TTX/STX by interacting with the TTX/STX binding site. On track (a), the work consists largely of electrophysiological studies of newly discovered or synthesized analogues of TTX and/or STX, utilizing the voltage-clamped preparation to study specific ionic conductances. This phase of the work is virtually complete. All reactive groups on the surface of the TTX and STX molecules have been touched through the examination of at least one representative analogue. Such refinements have led us to formulate the probable physical dimensions of the TTX/STX binding site, with 5 - 6 anchoring points for specific reactive groups in the toxin molecules. Three manuscripts describing this work is now in the press. This work has been covered in some detail in past quarterly and annual reports, and also in the final report, and, therefore, will not be repeated here.

On track (b), past attempts to synthesize new compounds have been hampered by the limited knowledge of potential reactive binding sites. Because of the new developments in recognizing the TTX/STX binding site, we are taking a new approach to this work. In the past year, our major effort has been focussed on the synthesis and biological testing of some of these new compounds, most of which show sodium-channel blocking properties. However, there are still problems with respect to their selectivity for sodium channel that need to be addressed.

Background. TTX and STX are important neurobiological tools because of their specific reaction with the voltage-gated sodium channel. Although the biophysical mechanism of the channel blockade has been studied exhaustively, the nature of the chemical interaction is poorly understood. The reason for the latter is that both toxin molecules have unique structural features which make them difficult to modify chemically. Past attempts at studies of structure-activity relations have not been successful, mainly because modifications of the structures tended to cause marked loss of channel-blocking activity. Since the mid 1970's, because of improved separation technology and better detection methods, a series of natural analogues of both TTX and of STX have been discovered. These analogues are usually only slightly modified from the parent toxin molecules, and often poesess measurable degrees of channel-blockade. Utilizing such analogues, and a few synthetically modified ones, I have identified some active groups in the toxin molecule, and, more importantly, some stereospecific similarities in two otherwise different molecules. During the contract years, several additional important analogues have been studied, leading ultimately to the formulation of the probable shape and size of the

TTX/STX binding site. This site is situated in a pocket 9.5 Å wide x 6 Å tall x 5 Å deep. Figure 1 shows a perspective view of a molecule of TTX (Fig. 1A) and also a molecule of STX (Fig. 1B) in the binding site. There are 5 - 6 anchoring site-points to interact with reactive groups in each toxin molecule, designated as sites a - g. Of these, sites a and d are anionic sites (probably

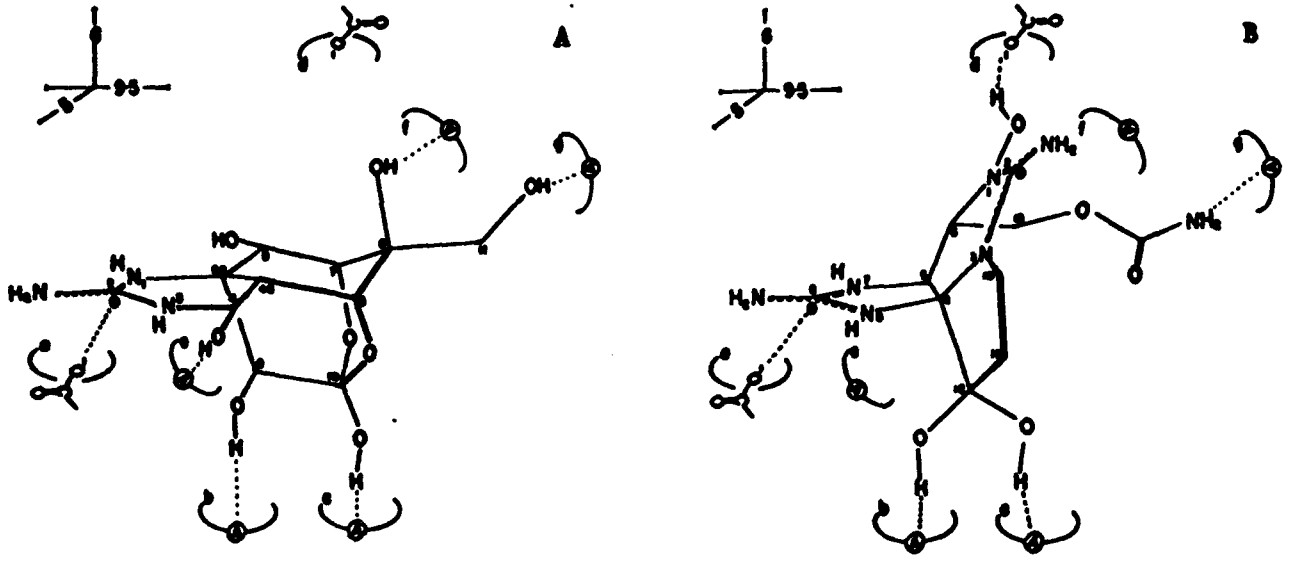

Fig. 1. Perspective views of tetrodotoxin (A) and neosaxitoxin (B) in the TTX/STX binding site. Scale in upper left corners is in Å units.

deprotonated carboxylate groups of amino acids) which form ion-pairs with the normally cationic guanidinium groups in the toxin molecules. The others are hydrogen acceptors which form hydrogen-bonds with hydroxyl groups on the surface of the toxin molecules. This view of the TTX/STX binding site explains a number of interesting features of the actions of TTX and/or STX analogues: (a) the equipotency of tetrodotoxin and chiriquitoxin (which has a large substituent on the basic structure of TTX); (b) the minor differences between the potencies of STX and gonyautoxins (which have large and strongly negative sulfate groups); and (c) the marked differences between the potencies of STX and the sulfocarbamoyl toxins which also contain sulfate groups.

Past attempts at synthesizing simpler compounds capable of mimicking or antagonizing TTX have focussed on satisfying binding to sites a, b and c. These efforts have proven to be very arduous and disappointing, because some of the promising structures, although identifiable by NMR spectra, could not be isolated in pure states for testing. When a fuller knowledge of all the reactive anchoring site-points became know, we embarked on synthesizing compounds which can interact with sites a, f, and g. Our efforts in the past year have focussed on this class of compounds.

In addition to such efforts, we have successfully modified TTX to a reactive intermediary, 11-oxoTTX, which is being used to produce a specifically labelled radioactive TTX, but also other potentially useful photoactivable marker substances. The specific aim of this work is clearly to try to locate the TTX/STX binding site with directly reacting compounds.

WORK DONE IN THE PAST YEAR

2-aminobenzimidazoles: The structures capable of binding to sites a, f and g have a basic structure of 2-aminobenzimidazole. To date, 12 compounds have been synthesized, and 9 of them have been tested biologically. With the exception of 2-aminobenzimidazole, which is available commercially, all the others were synthesized by Dr. B. Q. Wu in this laboratory. Some of the compounds have been described in the literature, but are not available; others are new (previosuly unknown). All these compounds have been characterized by elemental analyses and NMR spectra. Each of them was tested on the voltage-clamped frog muscle fiber for effect on the sodium and potassium channels. They have ED_{50} ranging from 0.5 to 10 mM. In addition, we also tested benzaldehyde (which had little effect up to 0.5 mM) and procaine (ED_{50} of 0.2 - 0.4 mM). Although all the 2-aminobenzimidazole compounds can block the sodium channel, they also affect, to varying degrees, the potassium channelin much the same way as procaine.

11-oxoTTX. This analogue of TTX differs from TTX in having $-CH(OH)_2$ in place of $-CH_2OH$ on C-11. As I have reported before, the oxidation of the primary alcohol group on C-11 to an aldehyde was postulated and expected, but never found. Khora and Yasumoto discovered 11-oxoTTX as a natural analogue in a puffer fish, Arothron nigropunctatus, in Micronesia, and showed that it was a hydrate of the elusive aldehyde. This discovery is of some significance, because 11-oxoTTX is an important intermediary for derivatizing TTX. It is superior to norTTX, because the latter exists only in an equilibrium mixture of varying proportions with two other compounds. We have invested heavily in making 11-oxoTTX synthetically, and I have reported on this before. The reaction is not easy, and the yield is still variable at around 25-35%. We have now identified another product in the oxidation reaction, 11-oxo 4, 9 anhydroTTX. If the right conditions can be found, we should be able to control the oxidation such that the proportion going to the anhydro state should be minimalized. These experiments are still in progress, but the identification of the byproduct is an important step for rational planning and improvement.

Photoactivatable derivatives of TTX. In spite of intriguing speculations on the structure of the sodium channel, and of the TTX/STX binding site on it, the only way to prove the structure is through direct identification of the amino acids in the binding site. For that, some covalent marker substance is needed, and there is no such substance in existence. Because we can make 11-oxoTTX, Dr. G. S. Wu has undertaken the task of synthesizing some photoactivatable derivatives based on 11-oxoTTX. His first task is to produce an appropriate photolable. The agent he chose is 4-azidopentafluronitrobenzene which should lead to nitrene formation upon irridiation with UV light >300 nm. This compound has been made and characterized. The second task is to couple this agent to 11-oxoTTX via ethylene diamine. By fluorescenc on TLC plate, TTX fluorescence can be detected, and the compound still needs to be isolated from other material in the reaction mixture. When it is sufficiently cleaned up, we will test its biological activity, with and without UV irridiation. We expect that if a covalent bond can be formed upon photolysis, then we should see irreversible blockade of the sodium channel. If the reaction is successful, then we will next move into biochemical work to collect the labelled site, in collaboration with Dr. Peter Kao of Stanford University Medical Center, who will continue with the sequencing work.